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Molecular techniques are better choice as compared to conventional methods for pathotypic characterization of Newcastle disease virus in poultry birds

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Newcastle disease (ND) has importance in the poultry industry across the world. Despite vaccination, outbreaks of ND have been reported from various parts of Pakistan. The purpose of current study was to characterize the Newcastle disease virus (NDV) through different methods i.e., molecular techniques and conventional methods and evaluate their efficiency. To isolate pathogenic NDV, samples were taken from the commercial poultry farms after an outbreak, while for the non-pathogenic NDV isolation commercially available Lasota vaccine was used. In the first step NDV was tested by F gene sequence through RT-PCR. The predicted sequence of the amino acid in fusion protein indicates the following motif ^{112}R-R-Q-R-R-F^{117} in pathogenic and ^{112}G-R-Q-G-R-L^{117} in non-pathogenic NDV strains. While in the second phase, the pathotypic characterization of NDV was done through conventional methods like Intracerebral pathogenicity index (ICPI) and Mean death time (MDT). The results from conventional methods were also in agreement with standard values. The findings of current study suggest that for the assessment of genetic nature and the rapid diagnosis of NDV; molecular techniques are better choice than conventional methods.

Keywords: Newcastle disease virus, poultry, Fusion protein, RT-PCR, Pakistan.

INTRODUCTION

Poultry is the largest group of birds which makes up over 30 percent of the animal protein. The production, however, is mostly because of commercial poultry, which represents only 20 percent of the overall poultry birds. Villagers rear poultry in developing countries to meet the demands for household meat, and as alternate sources of income (Abdisa and Tagesu, 2017). The meat of broiler is the most cost - effective source of animal protein in these countries and eggs has their own importance (Numan *et al*., 2005). White meat is a key source of protein in people's diets in most developing countries because of its cost effectiveness and high quality protein (Thomazelli *et al*., 2012).

World poultry is facing lots of challenges which includes: strong worldwide competition; changes in communal, administrative and consumer opinions of food safety and animal well-being; the increase of ecological protection issues; a steady escalation of feed cost; the outbreaks of new and unexpected diseases (Mottet and Tempio, 2017). Different types of pathogenic organism like bacteria, viruses and fungi cause diseases in poultry birds. These diseases produce low to high mortality in poultry flocks which leads to massive economic losses to farmers. Newcastle disease is one of them. World Organization for Animal Health has included ND in list "A" of diseases and its causative agent is NDV. This virus is included in genus Avulavirus of Paramyxoviridae family. Avulavirus infection leads to the wide range of clinical symptoms, which includes: asymptomatic to enteric signs and systemic illness with huge mortality (Putri et al., 2017). The NDV has the negative-sense, single stranded RNA genome which is composed of 15186 nucleotides. The genome is consisted of 6 genes that codes for hemagglutinin-neuraminidase (HN) , nucleoprotein (NP), matrix protein (M), fusion protein (F), Large polymerase protein (L) and phosphoprotein (P) (Steward et al., 1993).

Considering disease severity, NDV is classified in 3 pathotypes; lentogenic, mesogenic and velogenic strains. Lentogenic strains contributes to asymptomatic enteric infection of poultry birds. However, mesogenic and velogenic are considered as virulent strains and are responsible for major outbreaks of ND worldwide (Alexander *et al*., 2000).

Along with virus isolation, additional tests, like, Intravenous-Pathogenicity-Index (IVPI) , Mean-Death-Time (MDT) and Intracerebral-Pathogenicity-Index (ICPI) are used to

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characterize NDV. Molecular method such as; reverse transcriptase-polymerase chain reaction (RT-PCR) has been standardized to identify different pathotypes of NDV. There is a specific nucleotide arrangement at the cleavage site of the F gene. This specific sequence is the main reason to affect the pathotypic nature of the virus, so we use pathotype specific primers to detect NDV (Panda *et al.,* 2004). Therefore, this study was conducted to characterize the NDV circulating in this area. Molecular and conventional methods were used to identify the cleavage site sequence of F, so that pathotypic nature of ND virus can be determined. The results of this study provide us a comprehensive understanding of NDV in this area and evaluation of the methods used for its pathotypic characterization.

MATERIALS AND METHODS

***Tissue samples*:** To determine the pathotypic nature of NDV, the samples were taken from vaccinated commercial poultry flocks of district Faisalabad, after an outbreak during March 2019. The representative sample from dead "birds" including liver, spleen, proventriculus, brain and caecal tonsils were collected from different poultry farms having suspected ND cases and were instantly transported in the cold chain to the Institute of Microbiology, University of Agriculture Faisalabad. Samples were labelled and stored at -20°C till further use.

***Virus Isolation*:** By the use of sterile scissor, samples were chopped and triturated in pestle and mortar after that 20 percent w/v solution of the samples were made in phosphate buffer saline (PBS). The homogenized solution was transferred to sterile centrifugation tubes for centrifugation at 15000 rpm for 12 minutes at 4 °C to remove extraneous materials. The supernatant was collected, and antibiotics were mixed i.e. penicillin @ 2000 IU/ml and streptomycin @ 2mg/ml to inhibit bacterial contamination. After this supernatant was treated with chloroform (200 ul chloroform was added in 1ml of collected supernatant in the eppendorf tube and centrifugation was done at 12000 rpm for 4 minutes at 4°C) and inoculated within the allantoic cavity of 9-day old embryonated chicken eggs (ECEs). Virus was collected from eggs after 3 day's incubation at 37°C (Alexander, 1988). Heamagglutination (HA) test was done to measure the titer of the virus. While for the initial detection of viral isolates heamagglutination inhibition (HI) test was used. Positive and negative controls were also used at the same time with test samples to authenticate the results.

***MDT and ICPI*:** To perform the mean death time (MDT) assay, the allantoic fluid was mixed with PBS to make a 10-fold serial dilutions. For MDT assay, 9-days-old embryonated chicken eggs (ECEs) were used and 0.1 ml of the virus was injected in the allantoic cavity of eggs. The incubation was done at 37°C for 7 days. Each egg was examined twice a day to record the death time of embryo.

Similarly, to perform Intracerebral Pathogenecity index (ICPI), fresh allantoic fluid with HA titer of more than 24 (1/16) was diluted 1/10 in sterile saline. After that, day - old chicks were injected intracereberally with 0.05 ml of diluted virus. The birds were inspected carefully every 24 hours for a time being of eight days. On every inspection, the scoring of birds were done as 0, 1 and 2 for normal, sick and deceased (Kim *et al*., 2012) .

***RNA isolation and cDNA synthesis*:** The harvested virus from ECEs, was subjected to RNA extraction by TRIzol ™ reagent, by following the manufacturer's guidelines. After RNA extraction, cDNA was synthesized using Revertaid™ first cDNA synthesis kit (Thermoscientific,USA).

***Reverse Transcriptase PCR for fusion protein coding gene*:** The presence specific sequence in the F gene of NDV positive isolates was evaluated by reverse transcriptase PCR. The sequence of primers was NDV-FA (5^- TTGATGGCAGGCCTCTTGC 3^-), NDV-FB (5^- GGAGGATGTTGGCAGCATT 3^-) and NDV-FC (5^- AGCGTCTCTGTCTCCT 3^-). The two separate RT-PCR reactions were performed with different sets of primers, i.e. NDV-FA+NDV-FB and NDV-FA+NDV-FC for avirulent and virulent isolates respectively. As suggested by the producer, the reaction mixture of 25 µl was used. The RT-PCR protocol was designed in such a way that early denaturation was at 94°C for 5 min. After that 35 cycles were run with denaturation at 94°C for 30 seconds, primer annealing at 50°C (for both pairs) for 45 seconds, extension at 72 °C for 30 seconds and final elongation once at 72°C for 10 min. Then evaluation of the PCR product was done on 1% agarose gel in 6 ul volume (5 ul PCR product + 1 ul 6X loading dye). These samples were separated at 80 V for 45 minutes in 1 X TAE buffer stained with ethidium bromide along with 100 bp NDA ladder as marker. After that gel was visualized in Benchtop 2UV™ transilluminator.

***PCR product sequencing and analysis*:** Purified PCR products were sent to LabGenetix ™ Lahore, Pakistan for sanger sequencing. While alignment of sequence was done by using MEGA version 6.

RESULTS

Virus isolation was attempted from the different number of homogenized organ samples. Confirmation of the virus was done with the help of heamagglutination inhibition (HAI) and heamagglutination test (HA). Only six isolates were confirmed positive for NDV by these tests. The positive samples showed the agglutination sheet in the bottom of the plate in HA test while button formation was observed in HAI test. HA titers of these samples ranged between 64 and 512.

The positive samples were then cultivated in embryonated chicken eggs. In this 0.2 ml of the sample was injected in the allantoic cavity of eggs and incubated for 72 hours. Similarly, Lasota vaccine ™ was taken from the market and inoculated

in embryonated eggs for isolation of the virus. After chilling of eggs allantoic fluid was harvested. The mean death time of the viruses were 46 h for virulent and 99 h for the avirulent virus. Intracerebral pathogenicity index was 0.4 for the avirulent virus and 1.7 for the virulent virus.

After extraction of RNA, it was subjected to cDNA synthesis then RT-PCR was performed. In the beginning, by the use of "NDV-FA+NDV-FB" primers a 362 bp PCR product was obtained as shown in Figure 1. The sequence of PCR product is given and was submitted to GenBank NCBI with accession number MN909727.

While by using "NDV-FA+NDV-FC" primers a 254 bp PCR band of the F gene was obtained from virulent NDV as shown in Figure 2. Its sequence was submitted with accession number MN901804.

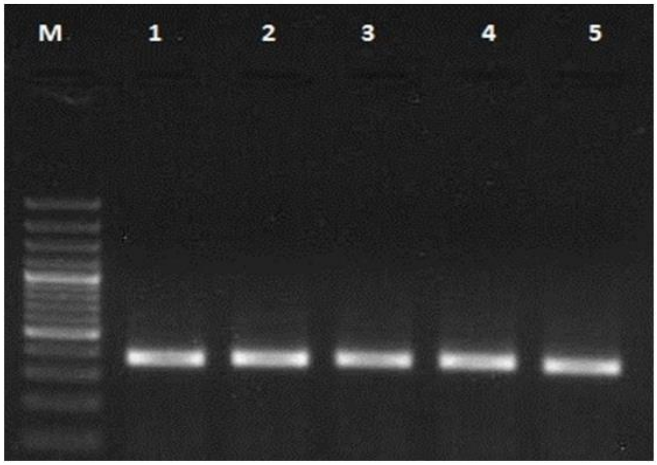

Figure 1. RT-PCR Product of non- virulent NDV F gene (362 bp) using FA+FB Primers, M= 100 bp DNA ladder, Lane: 1-5 Field samples.

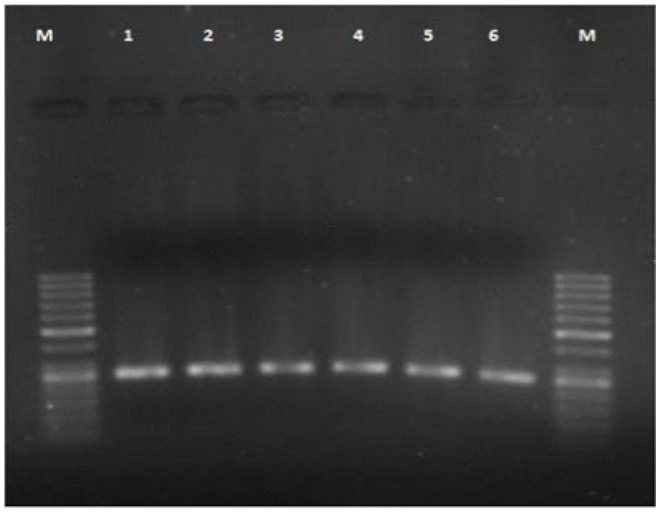

Figure 2. RT-PCR product (254 bp) amplified from virulent NDV using FA+FC Primers, M= 50 bp DNA ladder, Lane: 1-6 PCR Product.

DISCUSSION

Newcastle disease has importance in poultry industry due to its huge economic losses (Alexander, 2000). In this study, NDV was isolated from commercial poultry flocks of District Faisalabad, Pakistan. Out of collected samples, only six were found to be virulent when the sequencing of (F gene) RT-PCR product was done. The deduced amino acid arrangement in the field isolates were found in this order ^{112}R-R-R-Q-R-R-F^{117}, which agrees with the criteria of virulent NDV. While the commercial vaccine of lentogenic NDV (Lasota strain) was evaluated by RT-PCR. The predicted amino acid sequence shows motifs ^{112}G-R-Q-G-R-L$^{117.}$ This sequence was same as reported for the non-virulent NDV (De Leeuw *et al.,* 2003).

The fusion protein has an important role in the entry of NDV inside the host cell. Originally it is produced as F0 precursor. Host proteases convert this F0 precursor into F1 and F2. The world organization for animal health defines that "NDV" will be termed as virulent if it has an arrangement of amino acid in this order ^{112}R-R-R-Q-R-R-F^{117}; while for the non-virulent strain of NDV the sequence will be ^{112}G-R-Q-G-R-L^{117} (Aldous et al., 2003). Hence, the pathotypic characterization and diagnosis of NDV can be performed by RT-PCR then deducing the amino acid sequence.

Now a days, RT-PCR is being used for diagnostic purposes. This has led to the use of this technique in the diagnosis of NDV. The increasing knowledge in molecular techniques has enabled us to understand the molecular biology of Newcastle disease virus more comprehensively (Jestin *et al*., 1991). Many studies were designed to find out the option for the molecular diagnosis of NDV which can replace the traditional methods of MDT and ICPI. Most of the methods are RT-PCR based and amplified PCR product can be subjected to sequencing and the prediction of the amino acid sequence. This nucleotide sequencing can be used to analyze the cleavage site of the F gene (Kattenbelt *et al*., 2006).

In the present study, RT-PCR was used for identification and the pathotypic characterization of isolated viruses. In the first round, primers pair "FA+FB" was used to screen the NDV avirulent samples as result 362 bp band was obtained. While in second step, the primers pair "FA+FC" which represent the specific site of the F gene for virulent strains, was used to amplify 254 bp fragment in case of virulent virus (Elham *et al*., 2014). In the recent past, a similar type of study was designed for phylogenetic analysis of NDV in Pakistan during 2013, in NDV was isolated from the collected tissue samples. The predicted amino acid arrangement of the F gene shows motif with this sequence ^{112} R-R-R-Q-R-R-F^{117}. This amino acid sequence reveals that NDV isolates were velogenic in nature (Shabir *et al*., 2013).

Our results agree with previous studies reporting that virulent NDV is circulating in vaccinated flocks. It is important to note that samples were taken from commercial poultry farms of Faisalabad district during the specific period. This denotes

that more virulent strains of NDV may be circulating in the region. On the other hand, all commercial flocks were vaccinated with live or killed vaccines of NDV but still "outbreaks" occurred there. It means that NDV vaccine is not giving optimal protection against the challenge infection. To decrease the prevalence of NDV the current vaccines should be evaluated in terms of protection level (Rahmani *et al*., 2015).

For the proper controlling strategies of endemic disease in any country, the prompt and sensitive diagnosis is of prime importance. In routine practice, Newcastle disease is diagnosed on the basis of signs, symptoms, postmortem examination and some basic laboratory tests. While for pathotypic characterization of NDV; MDT, ICPI and IVPI are used. All the conventional techniques are time - wasting and sometime their results are not up to the mark. If we choose the RT-PCR option for characterization of NDV, it can overcome the problems of routine test used for this virus.

Overall, these results point out that in Pakistan, there are numerous velogenic strains that circulate and producing poultry epidemics. Nevertheless, it remains a concern whether these current NDV vaccines can produce a defensive immune response against the predominant field virus or not. In the current situation a well-known myth is gaining fame, that, the current NDV vaccines are unable to provide optimum protection against field isolates of the same virus. It is therefore important to rethink the criteria for the choice of a vaccine strain (Munir *et al*., 2012). Finally, it is concluded that RT-PCR is an easy and effective way to differentiate between a-virulent and the virulent strains of ND virus instead of MDT and ICPI. Rapid identification and Pathotyping of NDV is important for effective control of the disease. Further studies on the genetic, antigenic characteristics and the efficacy of commonly used NDV vaccines for protection against the NDV isolates are required.

Conclusion: It is concluded that RT-PCR is an easy and effective way to differentiate between a-virulent and the virulent strains of ND virus instead of MDT and ICPI. Rapid identification and Pathotyping of NDV is important for effective control of the disease.

***Authors Contributions statement*:** MH planned and executed the work; FM and ML helped the work and to compile the manuscript; MSM supervised the work and finally approve the manuscript

***Conflict of interest*:** The authors have no conflict of interest

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